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Arabidopsis AAL-toxin-resistant mutant *atr1* shows enhanced tolerance to programmed cell death induced by reactive oxygen species

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ABSTRACT

The fungal AAL-toxin triggers programmed cell death (PCD) through perturbations of sphingolipid metabolism in AAL-toxin-sensitive plants. While *Arabidopsis* is relatively insensitive to the toxin, the *loh2* mutant exhibits increased susceptibility to AAL-toxin due to the knockout of a gene involved in sphingolipid metabolism. Genetic screening of mutagenized *loh2* seeds resulted in the isolation of AAL-toxin-resistant mutant *atr1*. *Atr1* displays a wild type phenotype when grown on soil but it develops less biomass than *loh2* on media supplemented with 2% and 3% sucrose. *Atr1* was also more tolerant to the reactive oxygen species-generating herbicides aminotriazole (AT) and paraquat. Microarray analyses of *atr1* and *loh2* under AT-treatment conditions that trigger cell death in *loh2* and no visible damage in *atr1* revealed genes specifically regulated in *atr1* or *loh2*. In addition, most of the genes strongly down-regulated in both mutants were related to cell wall extension and cell growth, consistent with the apparent and similar AT-induced cessation of growth in both mutants. This indicates that two different pathways, a first controlling growth inhibition and a second triggering cell death, are associated with AT-induced oxidative stress.

Programmed cell death (PCD) is essential for a number of plant developmental processes and responses to pathogens [1]. Examples of developmentally regulated processes in which PCD is involved include embryo development, nucellar degeneration, maturation of tracheal elements and epidermal trichomes, formation of lace leaf shape, and leaf senescence [2]. Another type of PCD is represented by the hypersensitive response, a defense reaction in which plant cells in and around the site of pathogen infection die in order to physically restrict the spread of the pathogen [3]. While in the above examples cell death is beneficial and/or essential for plant development and survival, some necrotrophic pathogens can secrete toxins that cause cell death in healthy tissues so that the pathogens can feed on the dead tissues [4].

The fungal AAL-toxin triggers cell death through perturbations of sphingolipid metabolism in AAL-toxin-sensitive tomato [5]. The toxin inhibits ceramide synthase, a key enzyme in sphingolipid synthesis, which leads to accumulation of precursors and depletion of complex sphingolipids. Tomato plants sensitive to the AAL-toxin have a mutation in the *Asc* gene that is most likely a component of the ceramide synthase [6]. The *Arabidopsis thaliana loh2* mutant is more sensitive to the AAL-toxin than the wild type due to the knockout of a gene homologous to the tomato *Asc* gene [4]. Microarray analyses of AAL-toxin-induced cell death in *loh2* revealed induction of hydrogen peroxide-responsive genes and genes that are involved in the oxidative burst at early time points preceding visible cell death symptoms [4]. This indication of oxidative burst in AAL-toxin-treated plants was in agreement with previous studies demonstrating accumulation of reactive oxygen species in *Arabidopsis* plants treated with fumonisin B1 (FB1), an AAL-toxin analogue [7]. Moreover, a recently identified FB1 resistant mutant compromised in serine palmitoyl transferase, a key enzyme of *de novo* sphingolipid synthesis, failed to generate ROS and to initiate cell death upon FB1 treatment [8].

This paper describes a genetic approach carried out to isolate a mutant called *atr1* (AAL-toxin-resistant1) that survives AAL-toxin treatment, and its characterization in respect to reactive oxygen species-induced cell death. Microarray experiments of *atr1* and *loh2* under conditions that induce cell death only in *loh2* followed by bioinformatics analysis were carried out in order to identify genes with a potential role in the cell death process.

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Materials and methods

Plant material, isolation of mutants, growth conditions, stress treatments and cell death assessment. Forty-thousand seeds from *A. thaliana loh2* mutant, described earlier [4], were mutagenized with 0.1–0.3% ethane methyl sulfonate for eight hours. After extensive washing, the mutagenized seeds were planted on soil in pools and grown under standard greenhouse conditions (14 h light/10 h dark period, photosynthetic photon flux density 400 μmol m^{-2} s^{-1}, 22 °C and relative humidity 70%). Screening for resistance to AAL-toxin was done by plating the self-pollinated progeny seeds from M1 plants on growth media containing 40 nM of AAL-toxin and grown in a climate room under the following conditions: 60 μmol m^{-2} s^{-1}, 22 °C. AAL-toxin-resistant survivors, including *atr1*, were transferred to the greenhouse and seeds collected for further analysis. Light stress responses of *loh2* and *atr1* were evaluated by shifting in vitro-grown plants from 60 to 600 μmol m^{-2} s^{-1}. Chilling stress was applied by shifting one-week grown plants from 60 μmol m^{-2} s^{-1}, 22 °C to 240 μmol m^{-2} s^{-1}, 4 °C for 1, 2 and 3 days. Salt stress was applied by growing plants on media supplemented with 60, 80 or 100 mM NaCl. Abiotic stress tolerance was evaluated by measuring variable fluorescence Fv/Fm with Fluorcam 700 MF (Photon Systems Instruments, Brno, Czech Republic), fresh weight, and chlorophyll content. Assessment for tolerance to ROS-induced programmed cell death was done by plating *atr1* and *loh2* seeds on media containing either 7 μM aminotriazole (AT) or 0.5 μM paraquat and measuring the relative loss of fresh weight, chlorophyll, and visible cell death. Chlorophyll content was measured photometrically as previously described [9].

Isolation of RNA and microarray experiments. Samples for RNA isolation and microarray analysis were collected from *atr1* and *loh2* mutants grown on media with or without 7 μM AT four days after germination. One week after germination, this concentration of AT lead to mortality in *loh2* and no death symptoms in *atr1*. RNA was isolated using RNA Plant Mini Kit (Qiagen) as previously described [10]. Microarray experiments with two biological repetitions were performed in compliance with the MIAME standards [11]. The *Arabidopsis* 2 oligonucleotide array of Agilent Technologies was used, representing 21,500 genes. The labeling, hybridization, and data extraction were done at ServiceXS (The Netherlands) according to the instructions of Agilent Technologies as previously described [4].

Bioinformatics analysis. Datasets resulting from the microarray experiments were subjected to hierarchical complete linkage clustering using the Cluster/Treeview program [12]. Transcripts showing a minimum fivefold difference in expression in at least one experiment were clustered in two-dimensions: transcripts and mutants. Promoter regions of genes from clusters with similar mode of expression and known *cis*-regulatory elements of the genes were retrieved from the *Arabidopsis cis*-regulatory elements database, Ohio State University (http://arabidopsis.med.ohio-state.edu/). Search for new common *cis*-regulatory elements was done with the MEME/MAST system developed at Purdue University (http://meme.nbcr.net/meme/intro.html).

Results

Isolation of atr1

The *loh2* mutant of *Arabidopsis* is sensitive to AAL-toxin due to knockout of a gene involved in sphingolipid metabolism [4]. Forty thousand seeds from *loh2* were chemically mutagenized with ethane methyl sulfonate, germinated on soil, self-pollinated and the resulting progeny plated on AAL-toxin-containing media in order to isolate mutants that are more tolerant to AAL-toxin than the original *loh2* background. While the wild type *Arabidopsis* is resistant to 200 nM AAL-toxin, the *loh2* mutant develops cell death symptoms at 20 nM AAL-toxin already and 40 nM of the toxin leads to lethality. Thirty independent survivors were isolated using a concentration of 40 nM AAL-toxin as a screening threshold. The first one of them, named *atr1* (AAL-toxin resistant1), was selected for further analysis (Fig. 1). Genetic studies by crossing *atr1* with the wild type and studying the progeny indicated that *atr1* was recessive (data not shown). While *atr1* displays a wild type phenotype when grown on soil, it develops less biomass than *loh2* on growth media supplemented with 2% and 3% sucrose (Table 1).

Atr1 is more tolerant to ROS-generating herbicides

Earlier studies indicated that the AAL-toxin causes induction of ROS-associated genes and H_2O_2 accumulation that precedes the cell death [4]. To investigate this relation, the *atr1* was also tested for tolerance to PCD induced by reactive oxygen species (Fig. 2). The catalase inhibitor aminotriazole (AT) leads to H_2O_2 accumulation and subsequent cell death [13], whereas paraquat causes superoxide-dependent cell death [14]. Application of either AT or paraquat in plant growth media caused reduction in growth as measured by fresh weight loss (Fig. 2), reduction in total chlorophyll content (Fig. 2) and eventually death of *loh2*. However, *atr1* was more tolerant to both paraquat and AT than *loh2*, as estimated by the lack of cell death, smaller decrease in fresh weight and higher chlorophyll content. *Atr1* was asymptomous on 7 μM AT while the original *loh2* background died (Fig. 3). AT inhibits catalase activity in both *atr1* and *loh2* plants with the same efficiency (data not shown), suggesting that *atr1* may act downstream of hydrogen peroxide accumulation. As both AAL-toxin- and AT-induced cell deaths are light-dependent processes, we investigated the responses of *loh2* and *atr1* plants to light stress. Altering the light intensity from 60 to 600 μmol m^{-2} s^{-1} resulted in similar light stress responses in both mutants (data not shown). *Atr1* and *loh2* were also similar in their responses to chilling and salt stress (data not shown).

Gene expression analyses in loh2 and atr1 plants exposed to AT

While *loh2* plants on media supplemented with AAL-toxin or paraquat die at a very early stage without developing fully expanded cotyledons, AT treatment allow *loh2* plants to develop well-expanded cotyledons before they start dying and therefore constitute a very suitable system to analyze gene expression under cell death inducing conditions (Fig. 3). Microarray analyses of *loh2* and *atr1* under conditions that trigger cell death in *loh2* and no visible damage in *atr1* were carried out to identify genes specifically regulated in the two mutants. Both mutants were plated on medium without or with 7 μM AT, a condition that is eventually lethal for *loh2* and asymptomous for *atr1* (Fig. 3). Samples for microarray analysis were collected on the fourth day after germination, two days before the first visible cell death symptoms in *loh2*. The complete datasets are available as supplementary material. Genes (219) with an at least fivefold increase or decrease in AT-treated *loh2* or *atr1* plants compared to untreated plants were subjected to hierarchical complete linkage clustering analysis [12] and the results presented in Fig. 4. The most regulated genes from Fig. 4 are presented in Table 2. The clustering revealed four prominent clusters: genes upregulated in both *loh2* and *atr1* (cluster A), genes upregulated in *loh2* and downregulated or not regulated in *atr1* (B), genes downregulated or not regulated in *loh2* and upregulated in *atr1* (C), and genes downregulated in both mutants (D). The two biggest clusters in Fig. 4, namely A and D, consist of genes co-reg-

Fig. 1. Screening of *atr1* on medium with AAL-toxin. M2 seeds from ethane methyl sulfonate-mutagenized AAL-toxin-sensitive *loh2* plants were plated on plant growth medium supplemented with 40 nM AAL-toxin. The picture shows the survivor *atr1* one week after germination.

Table 1
Fresh weight of 10 day-old *loh2* and *atr1* seedlings grown on plant growth media supplemented with different concentrations of sucrose

	Sucrose concentration (%)			
	0	1	2	3
loh2	1.73 ± 0.43	3.26 ± 0.15	8.33 ± 0.44	8.1 ± 0.38
atr1	1.68 ± 0.09	3.17 ± 0.44	3.2 ± 0.43	3.83 ± 0.17

The fresh weight (mg) of 40 seedlings was determined for each genotype in each condition. Data represent the mean of one seedling ±SD obtained from three independent experiments.

ulated in both mutants, either coinduced (A) or corepressed (D). The cluster A comprise nitrate and ammonium transporters, peroxidases, transcription factors, a transposase, HSC70, WRKY and NAM family transcription factors, and a number of genes with unknown function. Twenty genes were exclusively induced in *loh2* and not induced or downregulated in *atr1* (cluster B), including four heat shock genes, two glycosyl transferases, a peptidylprolyl isomerase, and seven genes encoding for proteins with unknown functions. Seventeen genes were induced only in *atr1*, including two nicotianamine synthases, an allergen, and five genes with unknown functions. The cluster D of genes downregulated in both mutants contains two trypsin inhibitors, a protein kinase, arabinogalactans, expansins, xyloglucan *endo*-transglycosylases, a pectinesterase and proline-rich proteins.

In order to find common and specific *cis*-regulatory elements in the promoter regions of coregulated genes, promoters of those genes were analyzed for presence of known elements. Sequence binding sites for transcription factors of the WRKY, MYB, bZIP,

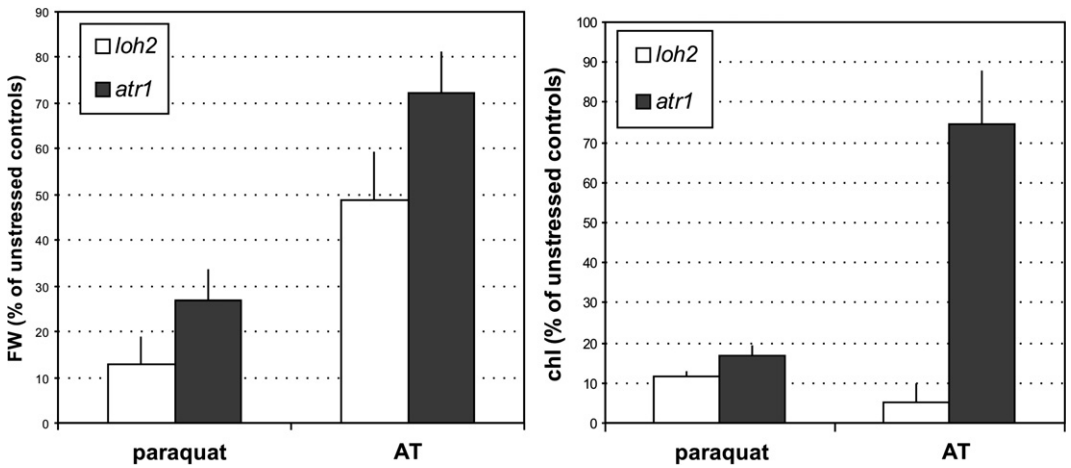

Fig. 2. *Atr1* is more tolerant to reactive oxygen species-induced cell death. Seeds from *loh2* and *atr1* mutants were plated on plant growth media supplemented either with 0.5 μM paraquat or with 7 μM AT in order to assess their tolerance to cell death induced by superoxide radicals or hydrogen peroxide, respectively. Data represents the loss of fresh weight (FW) or chlorophyll (chl) of *loh2* and *atr1* on media supplemented with paraquat or aminotriazole and compared with *loh2* and *atr1* grown without paraquat and aminotriazole (controls). Samples for the measurements were collected one week after germination. Data are means of three independent biological experiments ±SD.

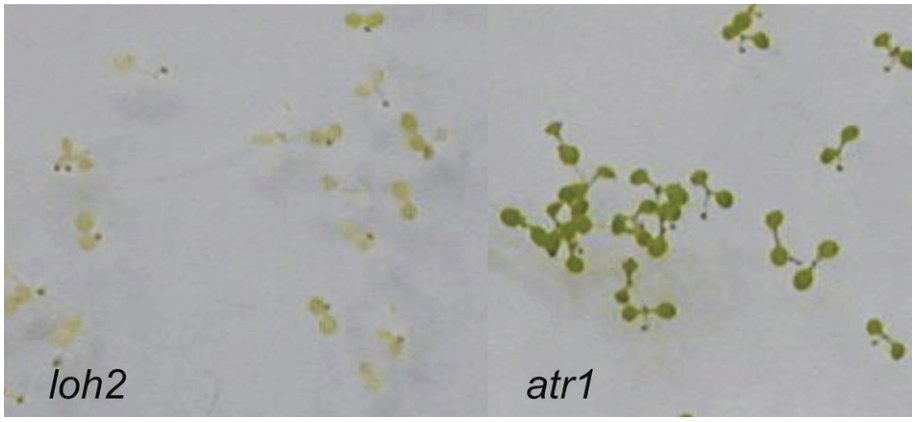

Fig. 3. *Atr1* is more tolerant to cell death induced by the hydrogen peroxide-generating catalase inhibitor aminotriazole. Seeds from the AAL-toxin-sensitive *loh2* and AAL-toxin-resistant *atr1* mutants were germinated on medium supplemented with 7 μM aminotriazole. The picture is taken ten days after germination. On the left, *loh2*; on the right, *atr1*. Under these conditions, all *loh2* plants died, while no visible cell death symptoms were observed in *atr1*.

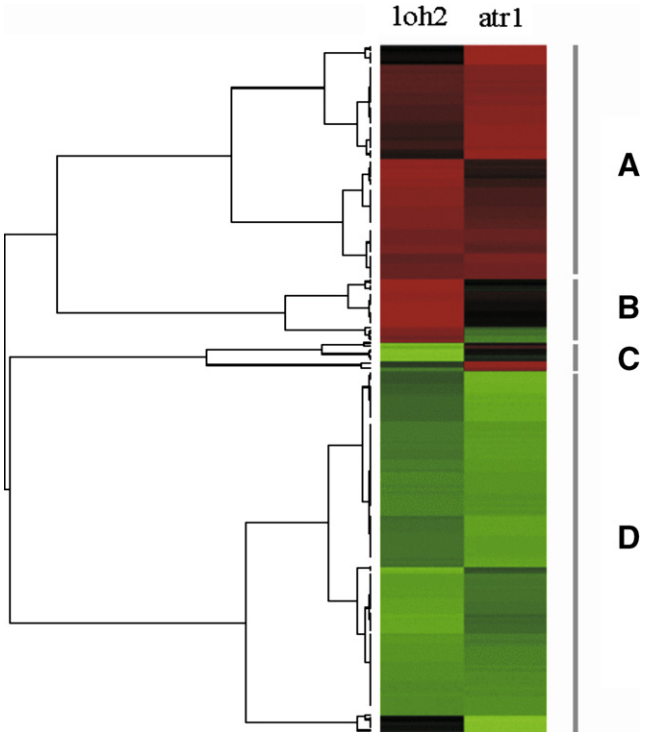

Fig. 4. Hierarchical complete linkage clustering of genes most altered in expression during AT-induced cell death. Samples from *Arabidopsis loh2* and *atr1* mutants grown on media without and with AT were collected on the fourth day after germination, two days before the cell death symptoms in *loh2*, and microarray analysis performed as described in materials and methods. The figure presents hierarchical average linkage clustering of 219 genes up or downregulated at least 5-fold by AT treatment in either *loh2* (first column) or *atr1* (second column), average from two biological replicates. Each row represents the expression profile of an individual gene. Red color indicates upregulation while green indicates downregulation. The color intensity corresponds to the extent of induction/repression. A, B, C and D on the right are the four main gene clusters.

GATA zinc finger family were frequent in many of the regulated genes, although none of those were present in all of the regulated genes. Some of the AT-regulated genes contained one or several *cis*-regulatory elements reported to be involved in H_2O_2 signaling, including the recently identified B-box and NRXe-2 elements [15,16]. A computational approach could not detect new common *cis*-elements specific for the gene clusters.

Table 2
Genes most altered in expression during AT-induced cell death

Gene	TAIR locus	*loh2*	*atr1*
High-affinity nitrate transporter	At1G08090	8.205	57.23
Peroxidase	At2G18150	5.425	8.86
PEP carboxylase	At3G42628	5.685	6.14
Transposase	At1G42110	7.445	5.76
HSC70	At5G02490	5.59	3.4
Isocytrate lyase	At3G21720	9.77	2.67
Hsp17	At3G46230	12.735	1.43
Hsp17.6	At5G12030	8.625	1.3
Peptidylprolyl isomerase	At5G48570	3.63	1.04
Allergen-like	At4G17030	1.61	4.72
Nicotianamine synthase	At5G56080	2.02	4.79
Xyloglucan *endo*-transglycosylase	At4G28850	−32.34	−56.05
Xyloglucan *endo*-transglycosylase	At2G18800	−30.595	−15.87
Extensin-like	At5G46890	−28.08	−23.74
Proline-rich protein	At2G33790	−27.72	−30.67
extA	At5G46900	−21.76	−20.9
Arabinogalactan AGP13	At4G26320	−10.43	−7.8
Cytochrome P450	At2G25160	−10.145	−4.23
Pectinesterase family	At5G04960	−8.41	−54.26
Putative protein	At5G62340	−7.985	−14.92
Hypothetical protein	At1G09720	−6.72	−7.18
Putative protein	At4G25250	−7.35	−6.79
Trypsin inhibitor	At3G04320	−6.29	−7.59
Hypothetical	At3G18295	−12.57	−1.26
Ripening-related	At5G51520	−1.08	−8.18

Arabidopsis thaliana loh2 and *atr1* mutants were grown on media without or with 7 μM AT and samples collected two days before cell death symptoms in *loh2*. Data are means of two biological replicates. Positive values indicate upregulated genes while negative values indicate downregulated genes.

Discussion

Taking advantage of a system for studying cell death triggered by AAL-toxin, second-site mutants more tolerant to AAL-toxin than the initial toxin-sensitive *loh2* background have been isolated. Earlier investigations revealed that AAL-toxin-induced cell death is connected with a burst of H_2O_2 and the activation of H_2O_2-responsive/generating genes [4]. Moreover, comparative transcriptome analyses of ROS-related experiments showed that the responses to AAL-toxin and AT treatments fall into a common gene cluster of photorespiratory H_2O_2 [12]. The isolation of *atr1* with enhanced tolerance to both AAL-toxin and ROS-generating agents is an additional genetic evidence for the link between AAL-toxin- and ROS-induced cell death. However, there is a clear difference between the symptoms of AAL-toxin-, AT- and paraquat-induced cell death,

suggesting that despite common production of H_2O_2 and similarities in gene expression, these treatments may activate different cell death signaling pathways. Recent discoveries of promoter regions and *cis*-regulatory elements specific for distinct types of ROS further support the notion for different ROS signaling pathways [16,17].

Both AAL-toxin- and AT-induced PCD are light-dependent processes. *Loh2* and *atr1* showed similar responses towards light stress, suggesting that the mutation is specific to cell death and not to light-dependent stress responses. The two mutants are also undistinguishable in their responses to other abiotic stresses tested. In contrast, *atr1* grows slower than *loh2* on growth media supplemented with 2–3% sucrose but not on media without or with 1% sucrose and on soil (Table 1). The reason for this phenomenon is unknown but it could be a 'trade-off' price for the cell death tolerance of *atr1*.

AAL-toxin-induced PCD is connected with both depletion of complex ceramides and accumulation of precursors, as inhibiting serine palmitoyl transferase abolishes the cell death [5]. Likewise, mutation of serine palmitoyl transferase in *fbr11* results in the absence of ROS burst upon FB1-treatment and the lack of cell death [7]. Thus, *atr1* seems to be different from *fbr11*. The inhibition of catalase activity by aminotriazole in both *loh2* and *atr1* suggests that the *atr1* mutation may be interfering with signal perception or/and transduction rather than hydrogen peroxide accumulation. Alternatively, the mutation may inactivate a gene essential for regulation or execution of the cell death program that is situated below the hydrogen peroxide perception and transduction.

Previous studies of the transcriptome during hydrogen peroxide-mediated cell death in wild type plants revealed similarities in gene expression with our datasets, for example induction of hydrogen peroxide-sensitive HSC70, transcription factors and peroxidases [10,18–20]. The results here reveal new H_2O_2-regulated genes, including a nitrate transporter, trypsin inhibitors, and protein kinases. Microarray analyses also revealed that *loh2* and *atr1* have very similar patterns of gene expression and only a small percentage of the transcripts are exclusively regulated in *loh2* or in *atr1*. Among them, two heat shock protein genes and a peptidylprolyl isomerase are induced only in *loh2*, an allergen-like gene is induced only in *atr1*, and a ripening-related gene is exclusively repressed in *atr1*. Heat shock proteins have diverse functions in plant biology and are rapidly induced under various conditions, including heat shock and oxidative stress [10,19,21]. While heat shock genes are extensively studied in plants, there is little functional data on plant peptidylprolyl isomerases [22].

Most of the repressed genes in *loh2* and *atr1* were related to cell wall metabolism, which in turn can govern cell growth and development. Expansins are primary wall-loosening factors that induce turgor-driven wall extension, while xyloglucan *endo*-transglucosylases as secondary factors reform the xyloglucan–cellulose wall structure, rendering it more responsive to the primary wall-loosening events [23]. The loose structure of the cell wall then allows growth of the cell. Repression of those genes, therefore, may inhibit plant growth and development. Indeed, growth inhibition that precedes cell death is clearly observed in *loh2* and to a lesser extent in *atr1*; however, the growth inhibition in *atr1* is eventually overcome and the plants continue to develop. In addition to extensins and xyloglucan *endo*-transglucosylases, the arabinogalactan proteins have also been implicated as regulators of cell growth and mediators of cell–cell interactions [24]. Downregulation of these genes is consistent with cessation of growth in both mutants and indicates that two distinct pathways may be activated by AT-induced oxidative stress: one controlling growth inhibition, active in both *loh2* and *atr1*, and another triggering cell death, executed in *loh2* but abolished in *atr1*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.08.056.

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